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Diabetes mellitus increases endothelin-1 gene transcription in rat kidney

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Background. Mesangial cell hypertrophy and increased extracellular matrix (ECM) contribute to mesangial expansion in early progressive diabetic nephropathy. Previous studies suggest that the growth factor endothelin-1 (ET-1) is not only upregulated in diabetes, but may mediate the effects of hyperglycemia on mesangial cell hypertrophy and ECM synthesis. In models of diabetes mellitus, the mechanisms underlying increased ET-1 peptide and mRNA remain unknown. Therefore, our purpose is to determine whether *ET-1* gene activity increases in kidneys of streptozotocin (SZT)-treated rats.

Methods. Male Sprague-Dawley rats were injected with either SZT or vehicle. Parameters including glucose, body weight, 24-hour urine volume, urinary protein, and urinary ET-1 excretion were recorded. All rats were sacrificed at 12 weeks postinjection. Prepro-ET-1 mRNA from whole kidneys was determined using both RNase protection and reverse transcription-polymerase chain reaction (RT-PCR). The abundance of ET-1 peptide in primary cultured mesangial cells was detected by indirect immunofluorescence following treatment with 5.6, 11.2, or 22.5 mmol/L D-glucose for 24 hours. Cellular ET-1 mRNA was measured using RT-PCR in control cells at time 0 and also following exposure to increasing concentrations of glucose for 24 hours. Rat mesangial cells were transfected with a luciferase reporter construct containing the rat ET-1 promoter (pET1.Luc), and relative ET-1 promoter activity was measured after a 24-hour exposure to 5.6 and 22.5 mmol/L of D- or L-glucose.

Results. After 12 weeks of hyperglycemia, diabetic rats gained less weight (344 ± 23.9 vs. 548.75 ± 15.08 g), had increased urinary volume (158.6 ± 24.32 vs. 8.38 ± 1.56 mL/day), and had marked proteinuria (101.7 ± 12.2 vs. 14.1 ± 2.8 mg/day) compared with controls. Total urinary ET-1 peptide increased 26.4-fold in diabetic versus control rats (17.5083 ± 5.405 vs. 0.6635 ± 0.343 ng/day). ET-1 mRNA extracted from whole rat kidneys was increased 2.1-fold in diabetic versus control animals. Primary cultured rat mesangial cells demonstrated a significant increase in immunofluorescence labeling of ET-1 peptide and ET-1 mRNA in response to increasing concentrations of glucose. Furthermore, transfected mesangial cells exposed to 22.5 mmol/L D-glucose showed a 1.6-fold increase in ET-1 promoter activity relative to those treated with 5.6 mmol/L glucose.

Conclusion. Glucose increases *ET-1* gene expression in the kidney of the SZT-treated rat model of diabetes mellitus. Furthermore, high glucose induces ET-1 expression in primary cultured rat mesangial cells and directly enhances ET-1 promoter activity. The greater relative increase in peptide compared with transcription suggests the potential participation of other mechanisms such as increased mRNA stability, protein stability, and/or enhanced translational efficiency.

Key words: progressive diabetic nephropathy, mesangial cells, glucose, vasoconstriction, extracellular matrix, amino acid peptide, hyperglycemia.

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Endothelin-1 (ET-1) is a 21-amino acid peptide that has a wide range of biologic activities that includes being one of the most potent vasoconstrictors known to date. ET-1 was initially isolated from the conditioned media of porcine aortic endothelial cells [1]. The current report focuses on the potential role of ET-1 in the pathogenesis of diabetic nephropathy. This complication is one of the most dreaded consequences of diabetes mellitus (DM) because of its association with high mortality [2]. The results of these studies are expected to provide new knowledge that will help us to understand better the etiology of this complication.

Several lines of evidence point to the participation of ET-1 in the pathogenesis of diabetic nephropathy. For example, extracellular matrix (ECM) expansion is one of the dominant histologic abnormalities found in diabetic nephropathy. The results of recent studies support the idea that ET-1 peptide may trigger increased production of several protein components of ECM [3]. The possibility that ET-1 underlies the histologic changes in the kidney of diabetic nephropathy is further strengthened by the fact that glomerular ET-1 mRNA is increased fivefold in rats rendered diabetic by treatment with streptozotocin (SZT) [4]. This finding suggests that ET-1 may play a key role in promoting mesangial expansion. Per-

haps ET-1 acts in an autocrine fashion and stimulates the cells that express this peptide to undergo changes associated with diabetic nephropathy. Another line of evidence supporting the involvement of ET-1 comes from the use of an ET-1 receptor antagonist in diabetic animals. The administration of these antagonists to the diabetic animals blocks the expected increases in glomerular mRNA that encode growth factors and selected ECM proteins. Members of this group of drugs also reduce urinary protein excretion [5, 6]. In other studies, angiotensin-converting enzyme (ACE) inhibitors, a class of drugs well known to halt the progression of early diabetic nephropathy and decrease glomerular *ET-1* gene expression in diabetic rats, were used [7]. Together, these findings point to a potentially important role of *ET-1* in the pathogenesis of diabetic nephropathy.

Despite previous studies showing enhanced expression of the *ET-1* gene in the setting of hyperglycemia, the molecular mechanism(s) underlying the increase remains unknown. The purpose of the current study is to determine whether increased ET-1 expression in glomeruli exposed to high concentrations of glucose arises from changes in gene activity. We postulate that hyperglycemia enhances transcriptional activity of the *ET-1* gene, which leads to increased glomerular levels of ET-1 mRNA and protein in the SZT-treated rat model of DM. In the present study, we examined the changes in ET-1 urinary peptide and renal prepro-ET-1 mRNA after 12 weeks of hyperglycemia in the SZT-treated rat model of diabetes. The actions of glucose were extended to primary cultured mesangial cells. These findings support the idea that glucose has a direct effect on activity of the *ET-1* gene.

METHODS

Animal studies

Male Sprague-Dawley rats weighing 200 to 225 g were purchased from the University of Calgary (Campus Breeders, Calgary, Alberta, Canada) and housed under standard temperature and lighting conditions. The animals were allowed free access to water and fed with regular rat chow (Purina, St. Louis, MO, USA). DM was induced in the rats by an intraperitoneal injection of 100 mg/kg body wt SZT (Upjohn, Kalamazoo, MI, USA) dissolved in sterile water. Control animals were given intraperitoneal injections of the same volume of vehicle alone. Twenty-four hours following injection, the glucose level in the rats was measured using a Medisense glucometer (Abbott Laboratories, Montreal, Quebec, Canada) with blood obtained by tail vein puncture. If the blood glucose was less than 13 mmol/L, the rats were given a second intraperitoneal injection of the same dose of SZT. None of the control or diabetic animals were treated with insulin.

Each animal was assessed at four-week intervals for total body weight, tail vein glucose, and 24-hour urinary volume. Aliquots of urine were stored at −70°C for total protein and ET-1 peptide determinations. The collection of a 24-hour urine sample was carried out by placing each animal in a metabolic cage. Total urinary protein was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA), and bovine serum albumin was used to generate the standard curve. Each sample was assayed in triplicate. Urinary ET-1 peptide concentration was measured using a specific radioimmunoassay kit containing a rabbit polyclonal primary antibody against ET-1 (#RIK-6901; Peninsula Labs, Belmont, CA, USA). The polyclonal antisera in this kit cross-reacted with various ET isoforms: ET-1, 100%; ET-2, 7%; and proET-1, 17%. ET-1 peptide was first extracted from the urine samples using pretreated C18 Sep-columns (#RIK-SEPCOL1; Peninsula Labs) according to the manufacturer's instructions, and was lyophilized and stored at −70°C. The lyophilized samples were resuspended in the recommended buffer solution, and the ET-1 concentration was determined using the radioimmunoassay technique. Total daily urinary ET-1 peptide excretion was calculated by multiplying the ET-1 concentration and the total 24-hour urine volume.

All rats were sacrificed 12 weeks after SZT injection. The kidneys were dissected from these animals, and 100 mg were snap frozen using liquid nitrogen and stored at −70°C for the extraction of RNA at a later date.

Primary culture of rat glomerular mesangial cells

Mesangial cells were obtained from glomeruli of 200 g male Sprague-Dawley rats as previously described [8]. Glomeruli were isolated by progressive sieving of chopped renal cortex using 250, 106, and finally 75 μm stainless steel sieves (Newark Wire Cloth Co., Newark, NJ, USA). The isolated glomeruli were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Life Technologies, Mississauga, Ontario, Canada) containing 5.6 mmol/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate, supplemented with sodium bicarbonate (3.7 g/L), HEPES (2.38 g/L), 1% penicillin-streptomycin (GIBCO BRL), and 20% fetal bovine serum (FBS; GIBCO BRL). Primary mesangial cells were identified by positive immunofluorescence for the intermediate filaments desmin and vimentin but were negative for cytokeratin. Mesangial cells from passages 13 to 17 were used for all experiments and were cultured to 90% confluence in six-well (35 mm) plates coated with 50 μg/mL human plasma fibronectin (Becton Dickinson, Mississauga, Ontario, Canada). For cells exposed to high glucose, an appropriate volume of a 1 mol/L solution of either D- or L-glucose was added to serum-poor media (0.5% FBS) to yield a final concentration of 22.5 mmol/L.

Immunofluorescence labeling of ET-1 in cultured mesangial cells

Cells were grown in DMEM supplemented with 20% FBS overnight prior to labeling. Primary cultured rat mesangial cells were grown on fibronectin-coated glass cover slips and were then treated with DMEM (0.5% FBS) containing 5.6, 11.2, or 22.5 mmol/L glucose for 24 hours. The cells were fixed and permeabilized with a solution containing a mixture of 3.7% formaldehyde, 0.25% glutaraldehyde, and 0.25% Triton-X in PEM buffer [160 mmol/L PIPES, 10 mmol/L egtazic acid (EGTA), 4 mmol/L $MgCl_2$, pH 6.9] for 10 minutes at room temperature. After washing three times with phosphate-buffered saline (PBS), the cells were treated with the reducing agent sodium borohydride, 1 mg/mL in PBS [9] for five minutes. The cells were washed again and then blocked with 1% goat serum for 10 minutes at room temperature. Rabbit polyclonal anti–ET-1 antibody (Peninsula Labs) was diluted 1:200 with PBS and added to each cover slip and incubated in a humid chamber for 60 minutes at room temperature. After washing, the FITC-conjugated secondary antibody (goat antirabbit IgG, H+L; Jackson ImmunoResearch Labs, West Grove, PA, USA) diluted 1:200 in PBS was added for 45 minutes at room temperature. Cells were washed and mounted on glass slides using mounting media containing 0.1% P-phenylene diamine and 50% glycerol in PBS. The FITC-labeled ET-1 peptide in cells was visualized using a Zeiss fluorescence microscope (Zeiss, Dusseldorf, Germany) with FITC excitation and emission wavelengths of 488 and 520 nm, respectively. Photographs were taken using Kodak Elite Chrome 100 film (Eastman Kodak Co., Rochester, NY, USA). Exposure times of one minute were performed for all slides. Final magnification was ×250. Each experiment was performed in triplicate.

Preparation of RNA

Total RNA was extracted from rat kidneys and primary cultured mesangial cells using TRIZOL Reagent (GIBCO BRL, Burlington, Ontario, Canada). The yield of total RNA was determined by measuring optical density at 260 nm, and purity was assessed by the ratio of optical densities at 260 and 280 nm (average ratio 1.7). For cell culture experiments, mesangial cells were grown in DMEM supplemented with 20% FBS overnight; 16 to 24 hours later, the media were changed to serum-poor DMEM (0.5% FBS) with D- or L-glucose added according to the glucose concentrations. The cells were exposed to 5.6 or 22.5 mmol/L of either D- or L-glucose in 0.5% FBS for 24 hours, and then cells were used for RNA extraction.

RT-PCR and RNase protection measurement of prepro-ET-1 mRNA

Total RNA (5 μg) extracted from kidneys of control and diabetic rats was reverse transcribed using 2 pmol of the antisense primer specific for rat prepro-ET-1 cDNA spanning nucleotides 717 to 740 (5′-ACTTTGCAA CTCGAAAGGAGGTCT-3′) and Superscript II RT (GIBCO BRL) in a final volume of 20 μL. A 2 μL aliquot of this product was used for the PCR reaction. A 554 bp product was amplified using Taq polymerase (94°C denaturation, 58°C annealing, 72°C extension, 30 cycles; Pharmacia, Uppsala, Sweden) and 10 pmol of specific primers for ET-1 (sense, ATGGATTATTTTCCCGT GATCTTC; antisense, ACTTTGCAACTCGAAAGG AGG-TCT), corresponding to sequences spanning 186 to 208 and 717 to 740, respectively, of the rat prepro-ET-1 cDNA. For the β-actin control, 5 μg of total RNA were reverse transcribed as described previously in this article using 2 pmol of a specific antisense primer (TAA CAGTCCGCCTAGAAGCATTTGCG). The same denaturing, annealing, and extension conditions used for the ET-1 PCR were used for the β-actin PCR. To 2 μL of the first strand reaction, the same antisense primer used for the RT reaction and the sense primer (CAT GAAGTGTGACGTTGACATCC) were added for 30 cycles. The reverse transcription-polymerase chain reaction (RT-PCR) protocol for cultured mesangial cells differed slightly in that 2 μg of total RNA and 1.25 μL (0.5 μg/μL) of oligodT (GIBCO BRL) were used instead of 5 μg total RNA and the specific ET-1 antisense primer used for tissue samples. The PCR products were run on a 1% agarose gel and stained with ethidium bromide. The relative band intensity was analyzed using quantitative scanning densitometry. The value for each ET-1 mRNA sample was standardized relative to the amount of β-actin mRNA signal obtained following 30 cycles of PCR for the same sample. RNase protection analysis was performed with 10 μg of total RNA from mesangial cells using the RiboQuant kit (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. The radiolabeled cRNA probe was synthesized by inserting the 554 bp ET-1 cDNA described previously in this article into pGEM-T and then digesting this construct with Xmn I. The 2239 bp fragment when transcribed with RNA polymerase Sp6 (Pharmacia) in the presence of α-[^{32}P]-UTP (800 Ci/mmol) yielded radiolabeled cRNA probe used in the RNase protection assay. Each reaction contained 10^6 cpm of radiolabeled cRNA. Autoradiograms were analyzed using quantitative scanning densitometry or a phosphoimager (Molecular Dynamics, Piscataway, NJ, USA).

DNA constructs

To clone the rat ET-1 promoter, we synthesized two oligonucleotide primers that flank the DNA fragment spanning −594 to +57 of the gene (sense, GTTTAAA AAAGACTTGGGGG; antisense, TGCAGGACAAG CGGAGC-AGA). These primers were used in a PCR reaction (94°C denaturing, 58°C annealing, 72°C extension, 30 cycles) with rat genomic DNA as the template.

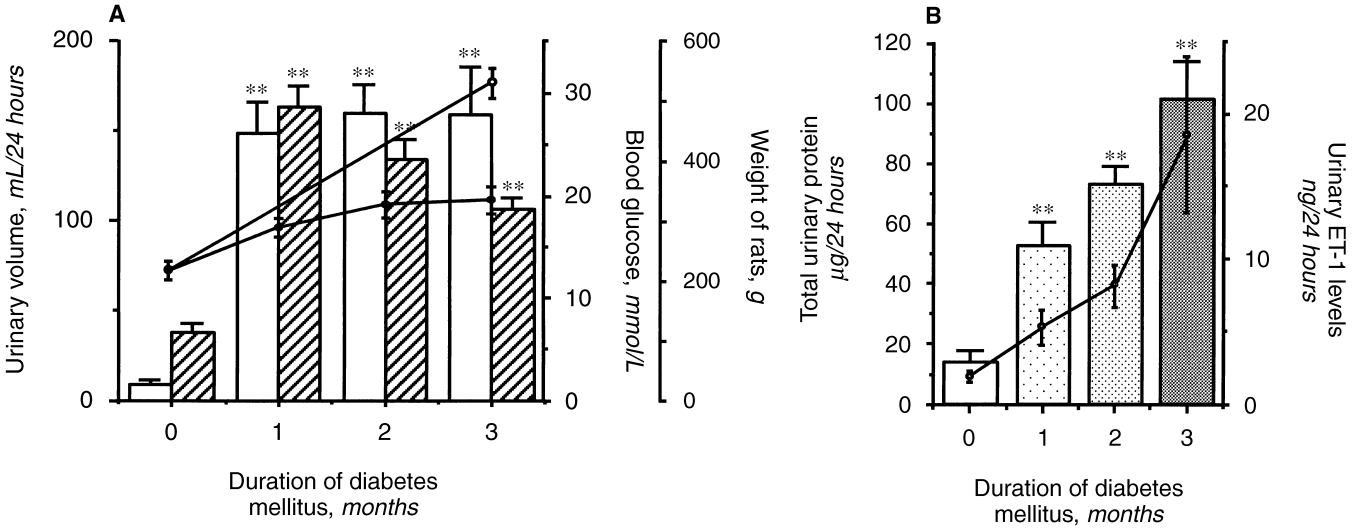

Fig. 1. DM increases urinary levels of ET-1. (*A*) The rise in blood glucose and urinary volumes in diabetic rats compared with controls over a time period of three months. Additionally, total body weight declined in the diabetic rats compared with controls. (*B*) The rise in total urinary protein and ET-1 peptide in the same animals. Each value reflects the mean ± SD. $^{**}P < 0.01$, according to analysis of variance of data.

The 651 bp product was purified, cloned into the pGEM-T (Promega, Madison, WI, USA) vector, and then sequenced using the standard dideoxy technique. Results demonstrated that it was identical to the rat ET-1 promoter from nucleotides −594 to +57 [10]. The resulting construct, pGEMT-ETP, was then used as the template for a subsequent PCR reaction using primers containing a Sac I restriction enzyme site at the 5′ end and Sac I and Kpn I sites at the 3′ end (sense, GGAGCT CGTTTAAAAAAGACTTGG; antisense, GGAGCTC GGTACCGCAGGACAAGCGGG). The 662 bp product was purified, cloned into pGEMT again, and cut with Sac I, and this purified fragment was then cloned into the luciferase reporter plasmid vector pGL3 (Promega) at the Sac I site. Colonies were screened for the resulting ET-1 promoter-containing plasmid (pET1.Luc, 5482 bp) in the forward orientation by assessing products of restriction enzyme digestion with Kpn I and Xba I.

Transient transfection and luciferase assay

Mesangial cells were subcultured in DMEM supplemented with 20% FBS and seeded onto fibronectin-coated, six-well (35 mm) plates at 24 hours prior to transfection; 1.5 to 2.0 × 10^5 cells were seeded per well, such that the cells would be 50 to 60% confluent at the time of transfection. One and a half micrograms of pET1. Luc and 0.5 µg of pSV2-CAT in 100 µL of DMEM free of serum and antibiotics were mixed with 5 µL of SUPERFECT Transfection Reagent (Qiagen, Inc., Mississauga, Ontario, Canada). The solution was incubated for 10 minutes at room temperature. A plasmid, pSV2-CAT, contains the bacterial reporter gene CAT driven by the SV40 promoter. Cellular uptake of this plasmid enabled us to monitor transfection efficiency using the CAT assay as described previously [11]. The 20% FBS-supplemented DMEM was aspirated from the cells; 1 mL of media was added to the DNA-SUPERFECT mixture before being applied to the cells. The cells were incubated with the complexes for two hours at 37°C and 5% CO_2. The complex-containing media were then aspirated. Cells were washed once with PBS, and fresh serum-poor (0.5% FBS) DMEM was added to each well. Transfected cells were allowed to recover overnight and were then exposed to either normal (5.6 mmol/L) or high-glucose (22.5 mmol/L) conditions for 24 hours. Cells were harvested and suspended in CCLR lysis buffer (Promega). A 20 µL aliquot of this lysate was used for determination of luciferase activity, and 10 µL were used for total protein determination (Bradford assay, Bio-Rad reagent). The luciferase activity from each measurement was expressed relative to the protein concentration of that sample. The relative ET-1 promoter activity was calculated by dividing the luciferase activity per µg protein by CAT activity (% conversion/µg protein/hour) in the same cells.

Statistical analysis

Final densitometric ratio data, as well as relative luciferase activity, were expressed as mean ± SD. Statistical significance was determined using analysis of variance. $P < 0.05$ was accepted as statistically significant.

RESULTS

Weight, blood glucose, and urine volume in diabetic rats

After 12 weeks of hyperglycemia, the diabetic rats gained less weight than the control animals (344 ± 23.9

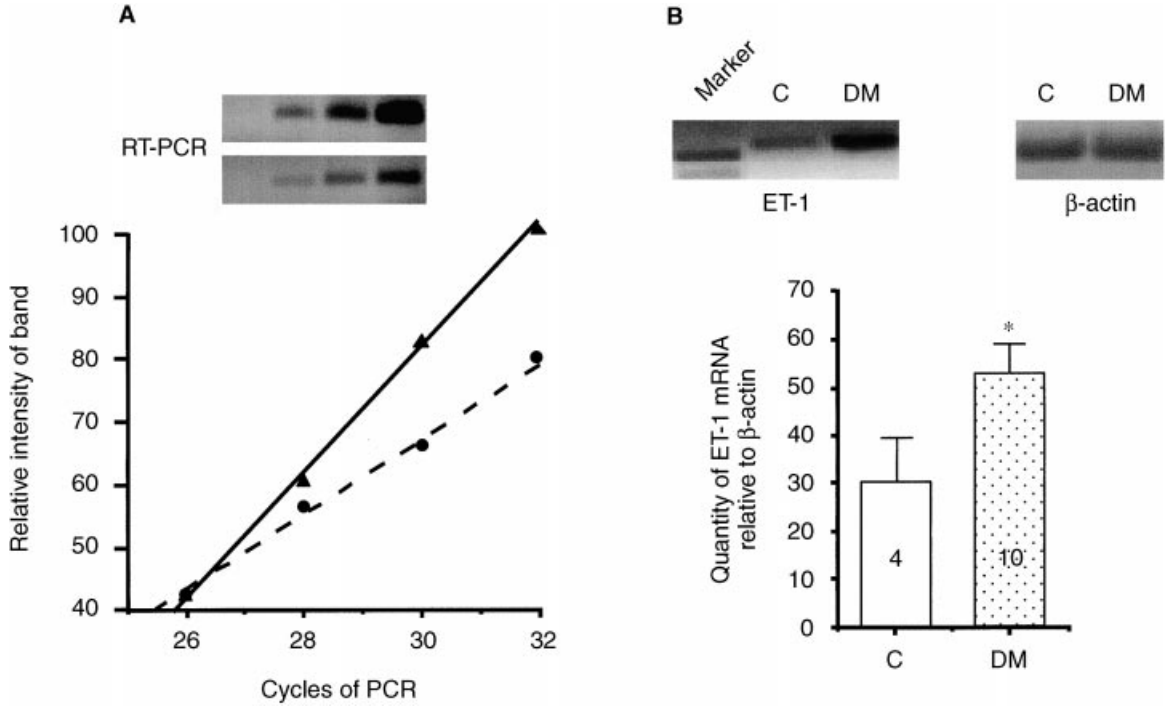

Fig. 2. DM increases renal ET-1 mRNA. (*A*) An ethidium bromide stained gels of RT-PCR products that reflect ET-1 mRNA abundance in total kidney RNA from a diabetic (upper) and control (lower) rat. The intensity of the bands was plotted as a function of cycle number and shows a linear rise in the product between 26 and 32 cycles of PCR. (*B*) The relative abundance of ET-1 mRNA in total kidney RNA from controls (4) and diabetic (10) rats. Each value reflects the mean ± SD. Analysis of variance showed a significant difference between the diabetic and control rats with $P < 0.05$.

vs. 548.75 ± 15.08 g), as demonstrated in Figure 1A. The mean blood glucose values in the diabetic animals were 28.8, 23.7, and 18.9 mmol/L at 4, 8, and 12 weeks postinjection of SZT, respectively. As expected, the diabetic rats also demonstrated a significant increase in urinary volume (158.6 ± 24.32 vs. 8.38 ± 1.56 mL/day for controls; Fig. 1A).

Diabetes mellitus increases urinary protein and ET-1 peptide

Figure 1B illustrates the incremental increase in 24-hour urinary total protein and ET-1 peptide excretion after 4, 8, and 12 weeks of hyperglycemia. Diabetic animals developed marked proteinuria (101.7 ± 12.2 vs. 14.13 ± 2.79 mg/day at 12 weeks), and total urinary ET-1 peptide was increased by 26.4-fold (0.6635 ± 0.343 vs. 17.508 ± 5.405 ng/day at 12 weeks) compared with controls.

Diabetes mellitus increases renal prepro-ET-1 mRNA

The results of the RT-PCR experiments using RNA extracted from the kidney of control and diabetic rats are illustrated in Figure 2. Representative photographs of the ethidium bromide-stained agarose gels are shown. Figure 2A shows the ET-1 signal of RT-PCRs products from a diabetic (top panel) and control (lower panel) rat kidneys following 26, 28, 30, and 32 cycles of amplification. Product amplification demonstrated that the use of 30 cycles for determining yield falls within the linear range of the reaction. The relative abundance of prepro-ET-1 mRNA was 1.8-fold higher in the kidneys of diabetic compared with that in control rats (Fig. 2B).

High glucose increases ET-1 peptide in cultured mesangial cells

Representative immunofluorescence images of ET-1 staining in mesangial cells treated with 5.6, 11.2, and 22.5 mmol/L D-glucose are illustrated in Figure 3A. Immunofluorescence labeling was uniform throughout the cytoplasm. The intensity appeared to increase as the concentration of glucose rose. Next, the abundance of ET-1 mRNA in the cultured cells was measured using RT-PCR following exposure to glucose concentrations of 5.6, 11.2, and 22.5 mmol/L. The results in Figure 3B and C showed that levels of the mRNA rose following exposure to increasing concentrations of glucose. In contrast, β-actin mRNA levels in the cells did not change regardless of the glucose concentration.

High glucose increases prepro-endothelin-1 mRNA in cultured mesangial cells

Mesangial cells were grown in DMEM with 20% FBS overnight, then exposed to serum-poor media (0.5%) containing 5.6 mmol/L D-glucose, 22.5 mmol/L D- or

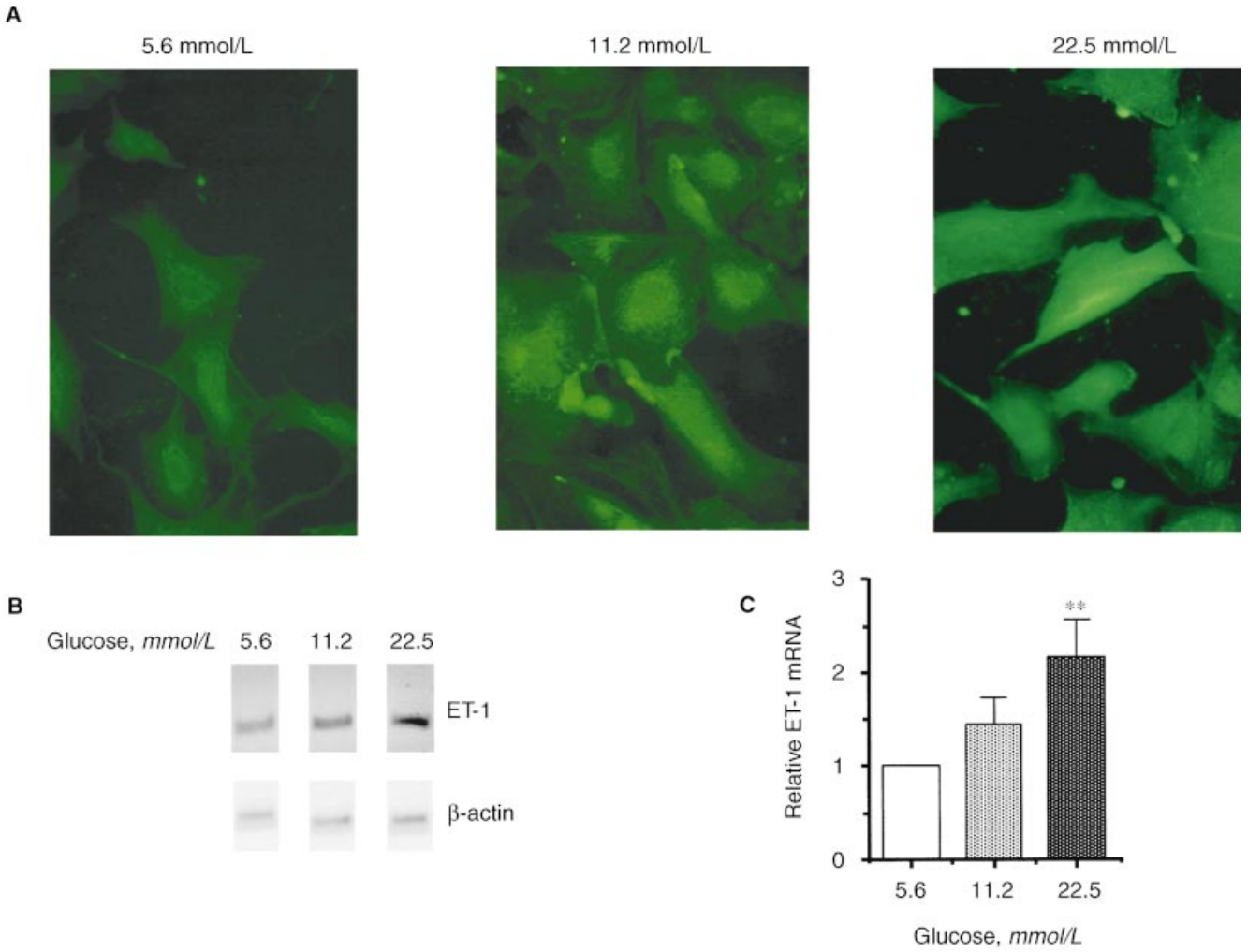

Fig. 3. Glucose increases ET-1 expression in primary cultured mesangial cells. (*A*) The increased ET-1 peptide, as detected by immunoflorescence, in primary cultured mesangial cells correlates with rising concentrations of glucose (5.6, 11.2, and 22.5 mmol/L) in the media. (*B*) Ethidium bromide-stained agarose gels containing products of RT-PCR that reflect the abundance of ET-1 and β-actin mRNA in primary cultured mesangial cells exposed to 5.6, 11.2, and 22.5 mmol/L glucose. (*C*) A graph showing the increase in ER-1 mRNA as a function of rising concentrations of glucose in the media. Each value reflects the mean ± SD. $*P < 0.05$; $**P < 0.01$, as determined by analysis of variance.

L-glucose (osmotic control), and harvested at 0 and 24 hours following exposure. The RNA extracted from these cells was used as template in RT-PCR to measure the abundance of prepro-ET-1 and β-actin mRNA (Fig. 4A). At time 0, there was no difference in the abundance of prepro-ET-1 mRNA from cells exposed to 22.5 and 5.6 mmol/L D-glucose. However, following 24 hours of treatment with 5.6 mmol/L D-glucose, the levels of prepro-ET-1 mRNA decreased compared with that in cells at time 0. In contrast, there was a 2.1-fold increase in prepro-ET-1 mRNA levels in cells treated with 22.5 mmol/L D-glucose relative to those exposed to 5.6 mmol/L glucose (Fig. 4B). The expression of prepro-ET-1 mRNA was not affected by 24 hours of treatment with 22.5 mmol/L L-glucose compared with the level in cells exposed to 5.6 mmol/L D-glucose at time 0. The levels of β-actin mRNA (Fig. 4A, lower panels) in the cells remained the same regardless of D-, L- or concentration of glucose used to treat the cells.

Since RT-PCR is semiquantitative, we used the RNase protection assay to measure ET-1 mRNA levels in mesangial cells exposed to 22.5 and 5.6 mmol/L glucose (Fig. 4C). The ET-1 cRNA probe is comprised of two bands (Fig. 4C, lane 1). The nonspecific hybridization of this probe to 10 μg of yeast tRNA was not significant. Consistent with the results of RT-PCR, total RNA from cells exposed to 24 hours of 22.5 mmol/L glucose protected radiolabeled ET-1 cRNA 1.8-fold more than in the 5.6 mmol/L-treated cells. The slightly higher fold increase of ET-1 mRNA between RT-PCR and RNase protection likely arises from differences in the techniques used. Together, these studies using two different assays of ET-1 mRNA show that abundance of this sequence increases following exposure to high levels of glucose.

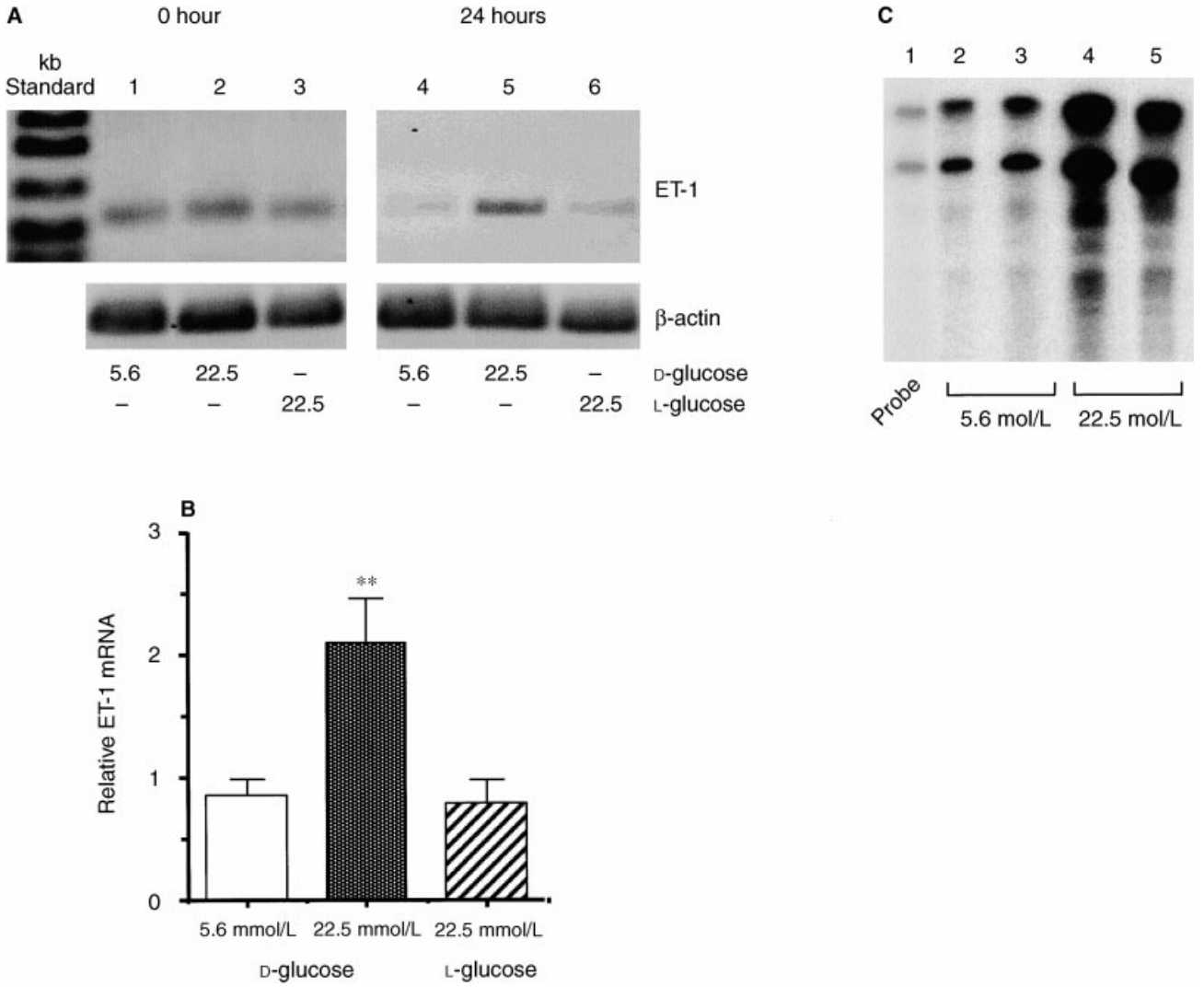

Fig. 4. Glucose increases ET-1 mRNA in mesangial cells. (*A*) RT-PCR products that reflect the levels of ET-1 mRNA from primary culture mesangial cells exposed to 5.6 or 22.5 mmol/L D-/L-glucose at 0 time, lanes 1 through 3. Lanes 4 through 6 show changes in the level of ET-1 mRNA following 24 hours of exposure to varying concentrations and isoforms of glucose. The RT-PCR product of β-actin mRNA appears below the ET-1 photographs, and the concentration of glucose is noted at the bottom of each lane. (*B*) The relative levels of ET-1 mRNA in primary culture mesangial cells exposed to 5.6 or 22.5 mmol/L D-/L-glucose for 24 hours. Each bar reflects the mean ± SD of at least three separate experiments. (*C*) An autoradiograph of results using RNase protection. Lane 1, probe alone; lanes 2 and 3, abundance of ET-1 cRNA protected in 10 μg of total mRNA from mesangial cells treated with 5.6 mmol/L glucose for 24 hours; lanes 4 and 5, abundance of ET-1 cRNA protected in 10 μg of total mRNA from mesangial cells treated with 22.5 mmol/L glucose for 24 hours.

Rat ET-1 promoter is responsive to high glucose

Figure 5A shows a schematic map of a plasmid that contains the rat ET-1 promoter fused to the luciferase gene. The products from digesting pET1.Luc with the restriction enzymes Kpn I and Xba I yielded fragments with sizes of 3076, 1742, 452, and 212 bp (Fig. 5B) and verified that the ET-1 promoter was inserted into the reporter construct in the forward orientation. Mesangial cells were transfected with pET1.Luc and exposed to 5.6 D-glucose or 22.5 mmol/L D- or L-glucose for 24 hours. Cotransfection of the pSV2-CAT (Fig. 5C) construct followed by assaying for CAT activity showed that the efficiency of transfection was the same among separate studies. ET-1 promoter activity increased 1.6-fold in cells treated with 22.5 mmol/L compared with that in cells exposed to 5.6 mmol/L D-glucose (Fig. 5D). Promoter activity was not significantly different between cells treated with 22.5 mmol/L L-glucose compared with those in 5.6 mmol/L D-glucose.

DISCUSSION

Diabetic nephropathy is one of the most serious consequences of DM. The need for further insight into the

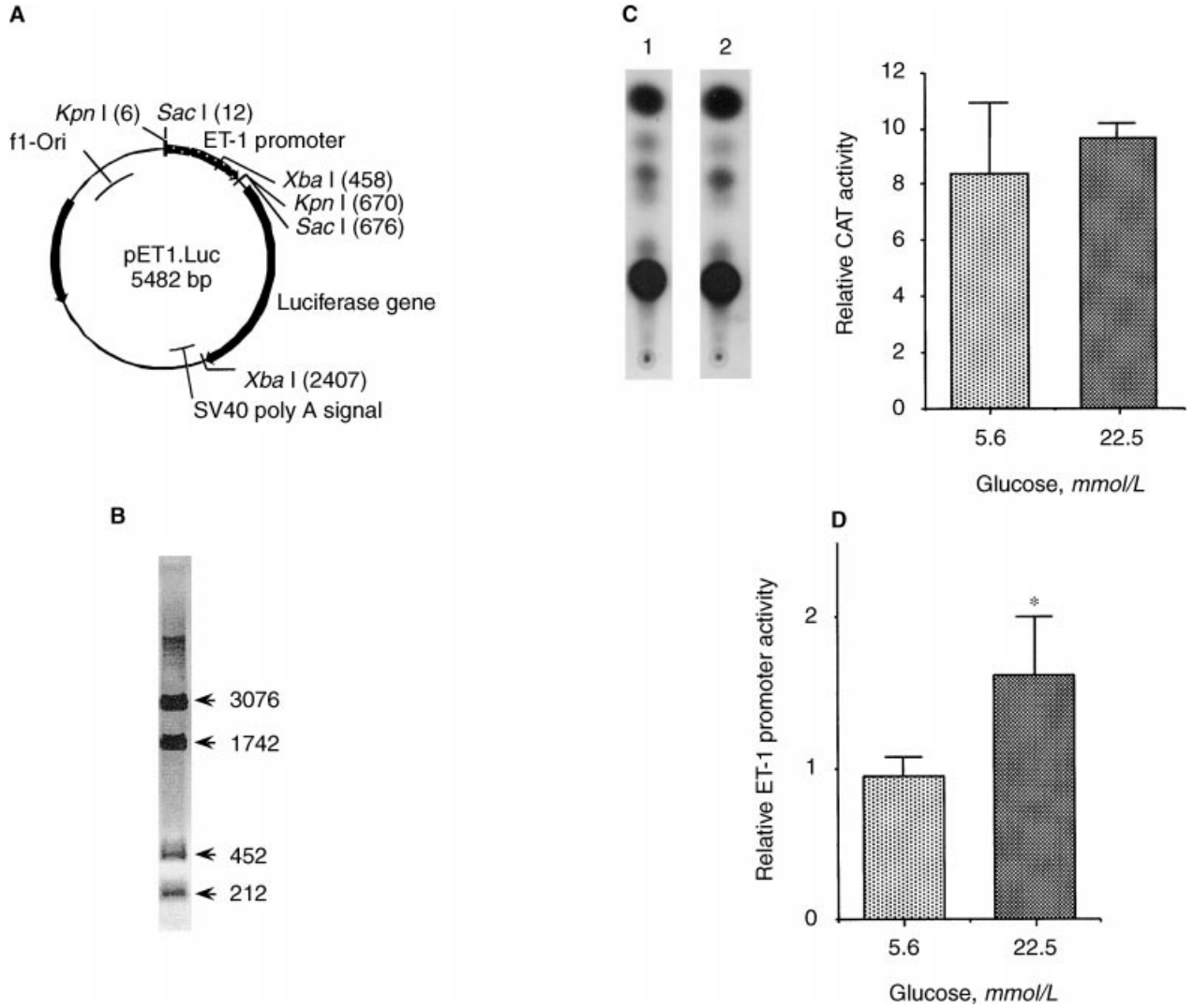

Fig. 5. Activity of ET-1 promoter is enhanced by glucose. (*A*) A schematic map showing the site of several restriction enzymes in pET1.Luc, a plasmid that contains the rat ET-1 promoter fused to the reporter gene luciferase. (*B*) An ethidium bromide stained agarose gel of fragments arising from the digestion of the pET1.Luc with Kpn I and Xba I. (*C*) Autoradiographs (left) and a graph (right) of CAT-activity from three separate cotransfections of cells with pSV2-CAT and pET1.Luc for each group of treatment with 5.6 or 22.5 mmol/L glucose. Each bar shows the mean ± SD of at least four separate experiments. (*D*) A graph showing increased relative ET-1 promoter activity (calculated as described in the **Methods** section) in primary cultured mesangial cells transfected with pET1.Luc followed by treatment with 5.6 or 22.5 mmol/L D-glucose. Each bar shows the mean ± SD of at least four separate experiments.

mechanism(s) underlying this disease provides the rationale for the current studies. To investigate the potential role of ET-1 in the pathogenesis of diabetic nephropathy, we used an accepted model of DM derived from rats injected with SZT. Consistent with the expected changes observed in rats with DM, the diabetic animals had higher blood glucose levels and urinary volumes but lower total body weight compared with euglycemic controls (Fig. 1A). The finding of proteinuria (>100 mg/day; Fig. 2) in the diabetic rats indicates the presence of glomerular dysfunction in these animals. Therefore, this model appears appropriate for our studies.

To investigate the potential role of ET-1 in the pathogenesis of diabetic nephropathy, we measured the abundance of the peptide in urine (Fig. 1B) from the diabetic animals and noted a 26-fold increase relative to that in euglycemic controls. Since ET-1 is a small peptide (molecular weight of 2492), increased filtration of ET-1 may account, in part, for the higher levels of the peptide in urine. In addition, some increase may be due to a reduced uptake of filtered ET-1 by the proximal tubules, but the 26-fold rise in urine content is unlikely explained by this mechanism alone. We postulate that the increase could arise from enhanced activity of the gene in glomerular or tubular cells. Therefore, we measured prepro-ET-1 mRNA (Fig. 2) in whole kidney. The mRNA was

1.8-fold higher in the diabetic rats relative to that in the controls. This finding is consistent with the results of Fukui et al, who demonstrated, using Northern blot analysis, a 3.8-fold increase of the mRNA encoding ET-1 in SZT-treated rats after 12 weeks of hyperglycemia. An even greater increase in ET-1 mRNA was evident after 24 weeks of hyperglycemia [4]. Additionally, Benigni et al have previously shown a twofold rise of ET-1 transcripts in diabetic versus control animals [6]. The same investigators used in situ hybridization to show that the rise in ET-1 mRNA was due in part to enhanced gene activity in the distal tubule epithelial cells of diabetic rats. Furthermore, glomerular staining for ET-1 was not detected in controls, but a significant increase was observed in the kidneys of diabetic rats.

The preceding results fit well with clinical data showing that patients with both type 1 and type 2 DM have increased urinary excretion of ET-1 [12, 13]. In these patients, the magnitude of the rise in urinary ET-1 correlates well with the severity of nephropathy, as manifested by albuminuria [14]. Plasma levels of ET-1 have also been reported to be elevated in humans with DM [15, 16]. Although we measured ET-1 levels in the plasma from both diabetic and control rats, a significant difference between the two groups was not detected (data not shown). The difference between humans and rats may relate to species specificity or sensitivity of the assay in detecting ET-1 levels.

Although the preceding results are consistent with published reports showing increased levels of ET-1 peptide and mRNA in the kidneys of diabetic animals, this information provides no further insight into the mechanism of the complication. One possible mechanism underlying the increase in ET-1 peptide and mRNA in DM is by enhancing transcription of the gene. To investigate this possibility, we focused the following studies on the mesangial cells because ET-1 expression in these cells is believed to act in an autocrine fashion to enhance ECM production leading to diabetic nephropathy [3, 17].

We chose to use primary cultured mesangial cells to study the regulation of ET-1 expression in response to one of the pathognomonic features of DM—high glucose—because these cells are believed to play a major role in the pathogenesis of diabetic nephropathy. High glucose has been shown to stimulate the production of ECM in mesangial cells directly [18]. Furthermore, these cells constitutively produce ET-1 [19], and secretion of the peptide from mesangial cells is stimulated by factors such as angiotensin II [20] and transforming growth factor-β (TGF-β) [19]. Both of these growth factors are upregulated in the setting of DM [21–24]. One hypothesis is that ET-1 may act as the focal point for integrating the actions of factors such as TGF-β and angiotensin II [25, 26]. Thus ET-1 is believed to play a central role in the pathogenesis of diabetic nephropathy. Added support for this idea comes from the studies of enalapril, an ACE inhibitor that slows the progression of diabetic nephropathy. This drug significantly reduces the levels of renal ET-1 mRNA in diabetic rats. Despite the fact that enalapril had no effect on increased glomerular mRNA levels for growth factors such as tumor necrosis factor-α (TNF-α), TGF-β, platelet-derived growth factor-B (PDGF-B), and basic fibroblast growth factor (bFGF), the reduction in glomerular ET-1 mRNA correlated with amelioration of proteinuria in the diabetic animals [7]. On the other hand, treatment of diabetic rats with an endothelin receptor antagonist significantly attenuated the increase in glomerular mRNA levels for ECM components, including α1(I), α1(III), and α1(IV) collagen chains, and laminin B1 and B2 chains. In addition, the expected increase in glomerular mRNA levels for TNF-α, PDGF-B, TGF-β, and bFGF arising from DM was attenuated by the ET-1 antagonist [5]. Together, these findings show that ET-1 is a key factor in the disease.

First, we tested whether *ET-1* gene expression is enhanced in mesangial cells exposed to high glucose, because alteration in the phenotype is a cardinal feature of diabetic nephropathy. Accordingly, we examined the effect of varying concentrations of glucose on ET-1 expression in primary cultured mesangial cells (Fig. 3). The relative expression of ET-1 peptide was measured using indirect immunofluorescence labeling of the peptide. Mesangial cells exposed to varying concentrations of glucose were labeled for ET-1 using a specific antibody. Results showed that the intensity of fluorescence varied directly with the concentration of glucose in the culture media. The most intense signal was noted in cells exposed to the highest concentration of glucose. Next, we used RT-PCR to measure the abundance of ET-1 mRNA (Figs. 3 and 4) in primary cultured cells exposed to 5.6, 11.2, and 22.5 mmol/L glucose. Cells treated with the higher concentrations of glucose for 24 hours had more ET-1 mRNA compared with those exposed to 5.6 mmol/L glucose. The increased abundance of ET-1 mRNA following exposure to 22.5 mmol/L glucose agrees with results of the RNase protection assay (Fig. 4C). The results of these studies showed two important points. First, the mesangial cell is one of the sites in the kidney where ET-1 is induced by glucose. Second, both ET-1 peptide and mRNA in these cells are enhanced following treatment with 11.2 and 22.5 mmol/L glucose compared with that in the control cells.

The preceding observations prompted us to measure ET-1 promoter activity in the mesangial cells. A construct containing 662 bp of the ET-1 promoter fused to Luc was created and then used as a reporter to assay the activity of the gene in mesangial cells. Results showed that 22.5 mmol/L glucose increased the amount of Luc activity by 1.6-fold after 24 hours (Fig. 5). This observa-

tion shows clearly that glucose has a direct effect on activity of the promoter. The activity of the promoter in the transfected mesangial cells fits closely with ET-1 mRNA levels in the kidneys of diabetic animals, noted previously in this article.

The ET-1 promoter was cloned and characterized in 1995 by Paul et al [10]. Sequence analysis identified putative binding sites for several transcription factors that could be involved in regulating rat *ET-1* gene expression. One of the most notable was an activator protein-1 (AP-1; c-fos/c-jun) binding site that spanned nucleotides −102 to −109 of the gene. Interestingly, a recent report suggests that hyperglycemia induced the binding activity of AP-1 in human mesangial cells [27]. An earlier investigation by Kawana et al revealed that the transcription factors GATA-2 and AP-1 were not only necessary for activity of the *ET-1* gene, but interacted synergistically to regulate its transcription [28]. Therefore, it is possible that the stimulatory effects of high glucose on ET-1 transcription may require the participation of AP-1, GATA-2, or both factors. Paul et al also found that angiotensin II had a dose-dependent stimulatory effect on ET-1 promoter activity in transfected bovine aortic endothelial cells [10]. They did not test the effect of high glucose on ET-1 promoter activity, and we plan to investigate whether there are synergistic effects of high glucose and angiotensin II on ET-1 promoter activity in future experiments.

Previous studies by others that investigate ET-1 expression in response to DM have examined either the peptide or mRNA, but not both. To our knowledge, the current report is the first to provide a complete study of renal ET-1 peptide and its corresponding mRNA. Furthermore, the study of these parameters plus transcription of the gene was extended to mesangial cells because we suspected the importance of this cell type in the pathogenesis of diabetic nephropathy. Thus, *ET-1* gene expression was measured in primary cultured mesangial cells by immunofluorescence labeling of the peptide. RT-PCR was used to measure the levels of ET-1 mRNA, and activity of the ET-1 promoter activity was determined by transient transfection, in response to varying concentrations of glucose. Yamauchi et al demonstrated that 22.5 mmol/L glucose significantly increased secretion of ET-1 from cultured bovine aortic endothelial cells [29]. ET-1 gene expression is also stimulated by insulin in endothelial cell culture [30], and angiotensin II stimulates ET-1 secretion in cultured rat mesangial cells [20]. To our knowledge, the concentration-dependent stimulation of mesangial cell ET-1 expression by glucose has not been reported. In addition, we also examined the molecular mechanism whereby high glucose stimulated increased ET-1 expression, and we found that glucose enhanced the activity of the promoter.

The significance of this finding is that glucose alone induces in mesangial cells the expression of the ET-1 peptide that is suspected of acting in an autocrine/paracrine fashion to trigger intracellular signaling. Perhaps the increased cellular ET-1 expression has an amplification effect on itself, or adjacent cells, to cause further stimulation of ET-1. Although we have not examined the pathways by which glucose stimulates ET-1 promoter activity, agents that induce ET-1 expression such as PDGF, vasopressin, and thrombin have been studied. They act via receptors coupled to the phospholipase C-mediated signaling system. Furthermore, mesangial cells with low intrinsic protein kinase C (PKC) activity or blocked by H-7, a PKC inhibitor, do not increase ET-1 expression in response to stimulation by phorbol ester or serum [31]. Substantial evidence supports a role for PKC contributing to the glucose-induced early signaling events that cause increased ECM accumulation by cultured mesangial cells [32, 33]. Amplification of the ET-1 response in mesangial cells may involve the activation of mitogen-activated protein kinase (MAPK), and this response is PKC dependent [34]. Together, these observations suggest an important role for PKC in the actions of ET-1. This may cause other perturbations such as ECM production [3], which is a hallmark of diabetic nephropathy.

The fact that we found a greater relative increase in ET-1 peptide (26-fold) compared with the mRNA and transcription rate in the animal studies suggests that other mechanisms such as increased mRNA stability, enhanced mRNA stability, increased translational efficiency, and/or protein stability could play a role in DM-induced increased expression of ET-1. Bitzan et al have shown that increased mRNA stability plays a significant role in increased ET-1 expression in their model system of thrombotic microangiopathy [35].

In summary, *ET-1* gene expression is increased in the SZT-treated rat model of diabetes and also in primary cultured rat mesangial cells exposed to high glucose. We have demonstrated that glucose increases activity of the ET-1 promoter in rat mesangial cells. Further studies are required to elucidate the molecular mechanism(s) by which high glucose stimulates ET-1 promoter activity. These topics will be the focus of future studies, which will give us further insight into the role of *ET-1* in the pathogenesis of diabetic nephropathy.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin-converting enzyme; AP-1, activator protein-1; bFGF, basic fibroblast growth factor; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGTA, ethyleneglycol-bis(beta-aminoethyl ether) N,N,N′,N′,-tetracetic acid; ET-1, endothelin-1; ETP, endothelin-1 promoter; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; SZT, streptozotocin; and TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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